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ORIGINAL ARTICLE

LC and LC–MS study for simultaneous determination of tramadol hydrochloride and ketorolac tromethamine in bulk and formulation with their major degradation products

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KEYWORDS

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Ketorolac;
LC–MS;
Stability

Abstract The present work was aimed to separate, identify and characterize the major degradation products of tramadol hydrochloride and ketorolac tromethamine. A rapid, specific and accurate stability indicating reversed phase liquid chromatographic method has been developed for simultaneous determination of tramadol hydrochloride and ketorolac tromethamine in bulk and formulation. The drugs were subjected to hydrolysis (acidic, alkaline and neutral), oxidation, photolytic and thermal stress, as per ICH guidelines. The separation, identification and characterization of major stressed degradation products were performed using high performance liquid chromatography combined with quadrupole electrospray ionization mass spectroscopy (LC/ESI-MS) on a C-18 column. Tramadol hydrochloride was found to degrade in acidic and oxidative conditions while ketorolac tromethamine undergoes extensive degradation under oxidative, UV and acid hydrolysis stress. From the mass spectral data, probable structures were assigned to the degradation products. The identified major degradation product for tramadol under acid stress may be 1-(3′-methoxy-3,4,5,6-tetrahydro-[1,1′-biphenyl]-2-yl)-N,N-dimethylmethanamine. Ketorolac tromethamine was also found to convert in to numerous degradation products under oxidative stress.

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1. Introduction

Tramadol hydrochloride (TRH) is (1*R*, 2R)-*rel*-2-[(dimethylamino)-methyl]-1-(3-methoxyphenyl) cyclohexanol.[1] It is a μ-opioid receptor agonist and centrally acting analgesic. Ketorolac tromethamine (KTM) is (±)-5-benzoyl-2,3-dihydro-1*H*-pyrrolizine-1-carboxylic acid, 2-amino-2-(hydroxymethyl)-1,3-propanediol[2] (Fig. 1). It is an analgesic and non-steroidal anti-inflammatory drug (NSAID). It acts by the inhibition of both cylooxygenase-1 (COX-1) and cylooxygenase-2 (COX-2) enzymes responsible for inflammation.

The combination formulation containing TRH and KTM is used as an analgesic for the short-term treatment of moderate to severe pain. It has higher analgesic efficacy than each of its components. Also, it has a faster onset of action and greater duration of effect.

KTM has been investigated either alone or in combination with other drugs by various methods like LC–MS/MS,[3] HPTLC,[4] LC–MS[5] and HPLC.[6–8]

Devarajan et al. have studied acid and base degradation of KTM alone using 0.5 N HCl and 0.5 N NaOH respectively with a reaction time of 10 min. HPTLC was used to separate one degradation product formed under both conditions.[4] Structure of this product was not elucidated. Salaris et al. have carried out alkali and acid degradation study under similar reaction conditions as reported by Devarajan et al.[5] They have used LC–MS for the identification of degradation products. No degradation product was reported under the experimental conditions probably due to very short reaction time.

Various techniques have been reported for the investigation of TRH which includes UV[9] and HPLC.[9–15] Only two reports exist involving the study of compatibility and stability of binary mixtures of TRH and KTM injection concentrate and diluted infusion using HPLC.[16,17] LC–MS is a sensitive technique used for the identification of other analytes in the presence of their degradation products.[18–21]

No study so far has been reported on characterization of degradation products of this combination under stress conditions prescribed by ICH Q1A(R2).[22] It is difficult to investigate multicomponent formulation along with degradation products. The challenge is to separate the drugs from the number of degradation products generated. This paper describes HPLC–UV and HPLC–MS method to determine TRH and KTM simultaneously in the presence of their degradation products. Relative stability of both drugs under various stress conditions has been assessed and identification of major degradation products has been performed with the help mass spectroscopy.

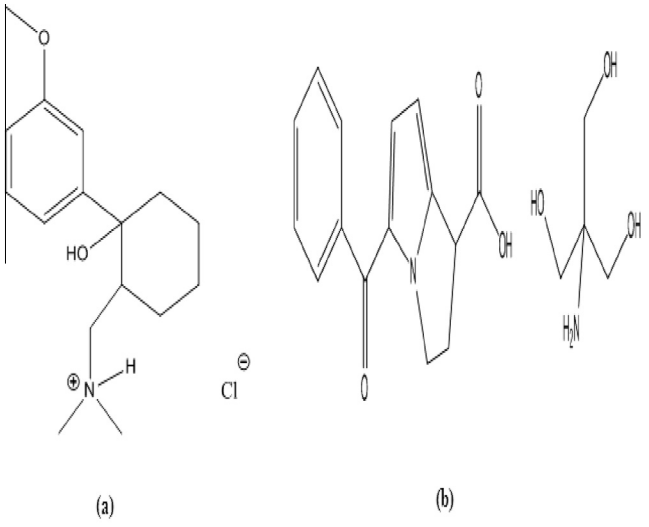


Figure 1 Structures of (a) TRH, (b) KTM.

2. Experimental

2.1. Chemicals and reagents

Pure TRH was obtained as a gift sample from Alkem Laboratories, Gujarat. KTM was provided as a gift sample by Dr. Reddy's Laboratory, Hyderabad. Methanol, acetonitrile and water were of HPLC grade (Merck, India). Formic acid (AR) was used for pH adjustment. Analytical reagent grade hydrochloric acid, sodium hydroxide and hydrogen peroxide used in the present study were purchased from S.D. Fine Chemicals (Mumbai, India). Voydol-C capsules manufactured by RAAM Laboratories were used for analysis. Each capsule contains 25 mg of TRH and 10 mg of KTM.

2.2. Instrumentation

2.2.1. HPLC–UV specifications

Dionex Ultimate 3000 HPLC (UV detection simultaneous determination at four wavelengths) with chromeleon software version 6.8 SR 10 build 2818 equipped with column oven and autosampler was used. Detection wavelength was set at 270 nm.

2.2.2. HPLC–MS specifications and chromatographic conditions

Dionex Ultimate 3000 HPLC (UV detection simultaneous determination at four wavelengths) with chromeleon software version 6.8 SR 10 build 2818 equipped with column oven, online degasser and autosampler was used. Applied biosystems MS (MDS SCIEX, 4000 Q TRAP) equipped with Turbo V electrospray ionization source (ESI) and analyst version 1.4.2 software were used for data acquisition and processing. The samples were infused into the mass spectrophotometer from HPLC system through ESI interface. The optimized parameters are given in Table 1. The samples were separated on a Neosphere C-18 column (4.6 internal diameter, 250 mm length, 5 μm particle size, Hexon Laboratories Pvt. Ltd.). The mobile phase composition was water: methanol: acetonitrile (53:23:24, v/v/v) with 0.5% of formic acid. The mobile phase flow rate was 1.0 ml/min and the detection wavelength was 270 nm. Injection volume was 25 μl.

Other equipments used were hot air oven (Dolphin, PPI unix96), pH meter (HANNA, HI 2211) and analytical balance (Shimadzu AUX 220, Japan).

2.3. Selection of analytical wavelength

Spectra of TRH (20 μg mL^{-1}) and KTM solution (10 μg mL^{-1}) were obtained separately using double beam UV Visible spectrophotometer (Shimadzu UV-1800).

Table 1 Mass specifications.

S. no.	Parameter	TRH	KTM
1	Source temperature	600 °C	600 °C
2	Mass range	50–500 amu	50–500 amu
3	Ionization mode	Positive ion mode	Negative ion mode
4	Declustering potential	+18 V	−10 V
5	Entrance potential	+10 V	−10 V
6	Ion spray voltage	5500 V	5500 V
7	Sheath gas/nebulizing gas and desolvation gas	Nitrogen at 50 psi	Nitrogen 50 psi
8	Curtain gas velocity	Nitrogen, 30 psi	Nitrogen, 30 psi

3. HPLC method development

Literature survey revealed the use of phosphate buffer, triethyl amine and acetonitrile as a mobile phase. Hence, it was decided to develop a mobile phase compatible with MS with minimum amount of organic modifiers. Numerous mobile phases in various ratios were tried so as to obtain well resolved peaks of drugs from the degradation products. The selection of mobile phase was made on the basis of peak shape and resolution between drug and degradation products.

The mobile phase consisting of water:methanol:acetonitrile (53:23:24 v/v/v) with 0.5% formic acid was prepared. It was filtered through a 0.45 μ membrane filter and sonicated for 15 min before use.

3.1. Forced degradation studies

Stress degradation studies were carried out on the bulk drug singly and in combination. It was also extended for formulation to study the presence of additional degradation products that may arise from drug excipient interaction. TRH, 31.25 mg and KTM, 12.5 mg were weighed and transferred to 25 ml of water leading to a concentration of 1250 μg mL^{-1} of TRH and 500 μg mL^{-1} of KTM. An aliquot (5 ml) of this solution was mixed with 5 ml of stressor (2 N HCL/2 N NaOH/water/3% H_2O_2). The solutions were heated at 90 °C for 5 h. Similarly, 5 ml of this solution was exposed to short wave UV light in UV cabinet. After stress testing, the acid solution was neutralised using 5 ml of 2 N NaOH. Base solution was also neutralised using 5 ml of 2 N HCL. The solutions were further diluted to 50 ml with mobile phase. Solution containing formulation was filtered before dilution.

Solid state photolytic studies were performed by exposing thin layers of drugs to short wave UV light for 12 h and diluted as described above. Drugs in the solid state were also exposed to dry heat at 80 °C for 8 h. After these treatments, all solutions and blanks were filtered from 0.25 μm nylon syringe filter for injection into HPLC system. The blank spectra were subtracted from the analyte spectra during mass investigation.

3.2. Assay of formulation

A powder equivalent to 31.25 mg of TRH and 12.5 mg of KTM was weighed accurately and dissolved in 20 ml of mobile phase and sonicated for 15 min and volume was made to 25 ml with mobile phase resulting in 1250 μg mL^{-1} solution of TRH and 500 μg mL^{-1} solution of KTM. The solution was filtered through Whatman filter paper No. 41. An aliquot (2.5 ml) of the solution was further diluted to 50 ml resulting in a concentration of 62.5 μg mL^{-1} for TRH and 25 μg mL^{-1} for KTM. Sample solution prepared individually six times was analyzed under chromatographic condition described above. The area under the curve of each peak was measured at 270 nm. The amount of each drug present in the sample solution was determined using calibration curves.

3.3. Stability of sample solution

The drug solution containing 62.5 μg mL^{-1} of TRH and 25 μg mL^{-1} of KTM were injected on column at defined intervals for 8 h and peak area was noted.

4. Method validation

Method was validated as per the ICH guidelines.[23] The following parameters were checked:

4.1. Linearity

TRH, 31.25 mg and KTM, 12.5 mg were weighed and transferred to a 25 ml volumetric flask containing 15 ml of HPLC grade water. The solution was sonicated for 5 min and volume was made up to 25 ml with HPLC grade water. The resulting stock solution containing 1250 μg mL^{-1} of TRH and 500 μg mL^{-1} of KTM was further diluted with mobile phase

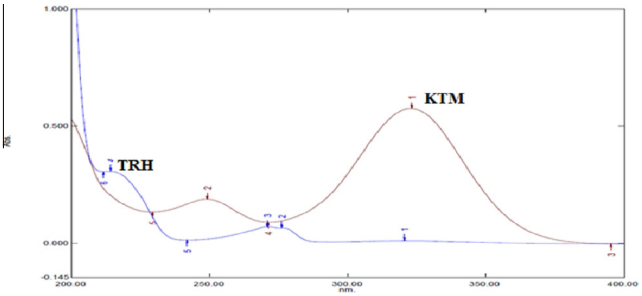


Figure 2 Overlay of absorption curves of TRH solution (20 μg/ml) and KTM solution (10 μg/ml).

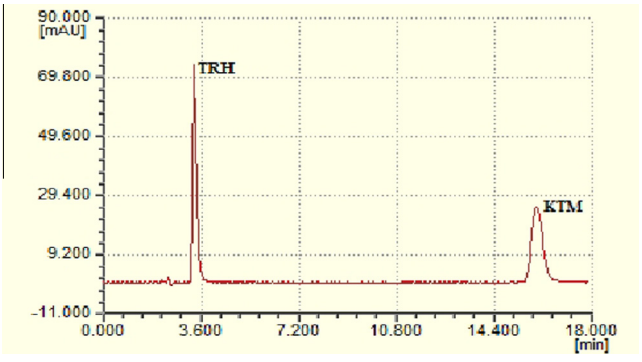
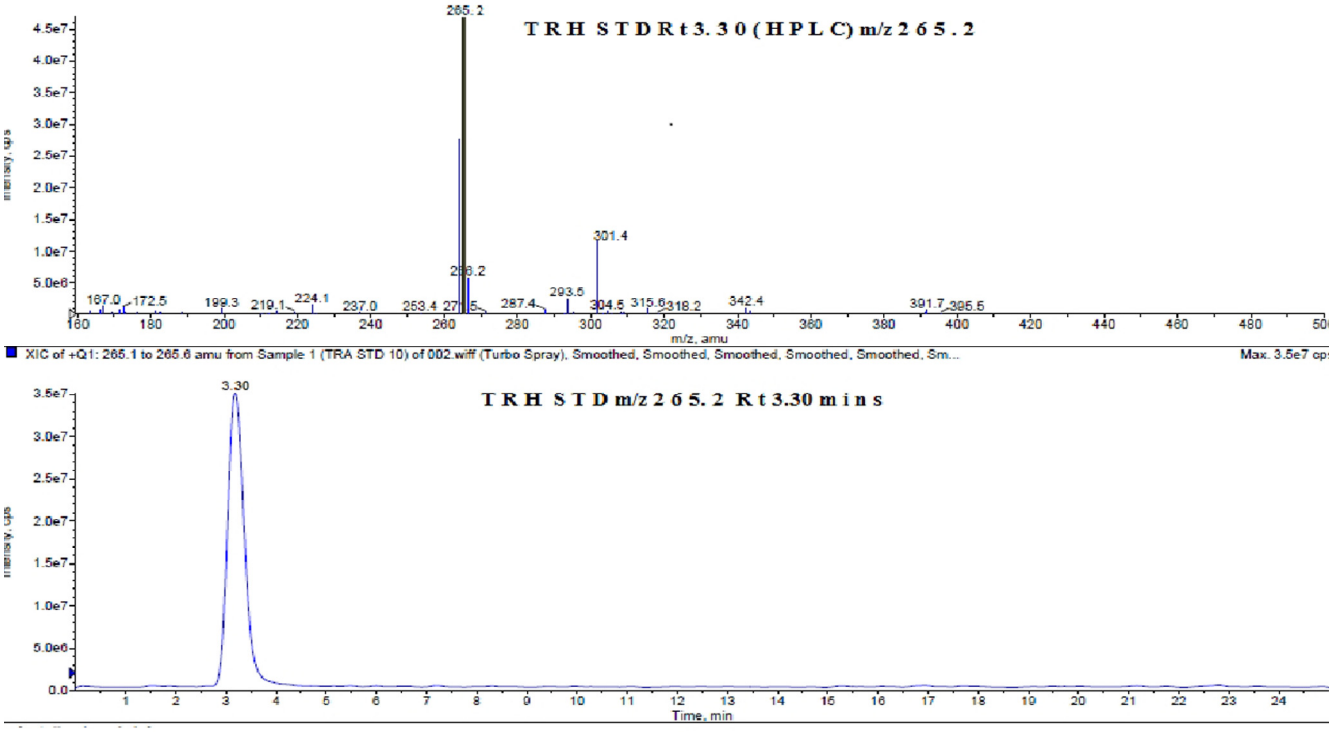
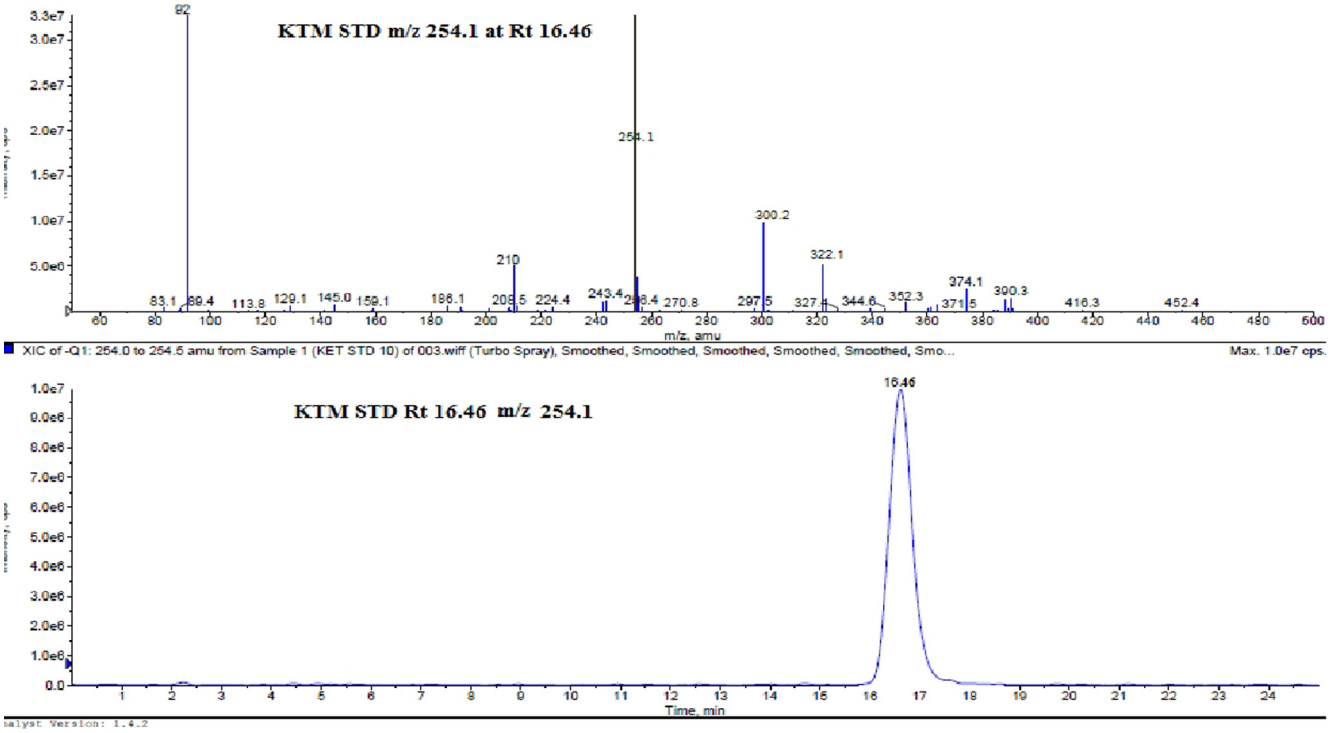


Figure 3 Chromatogram of TRH and KTM.

to get mixed standards in the concentration range of 12.5–125 μg mL^{-1} for TRH and 5–50 μg mL^{-1} for KTM. The solutions were analyzed for peak area. The whole procedure was repeated thrice from weighing of drugs. The average of peak area of three reading was calculated and was plotted against concentration to obtain calibration curve. The % RSD of peak area after independent analysis carried out thrice at each concentration level was considered to establish range.



(a)



(b)

Figure 4 LC–MS chromatogram of (a) TRH and (b) KTM.

Table 2 Stability of sample solution.

Duration	Peak area			
	1 h	2 h	4 h	8 h
TRH	38406.9	38705.1	38703.4	38684.3
KTM	37878.1	38713.8	38386.4	38268.4

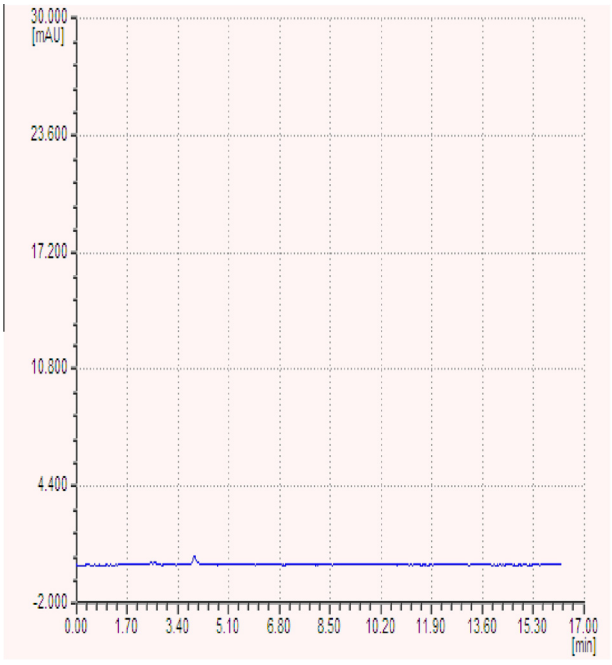


Figure 5 Blank chromatogram.

The correlation of determination (r^2), y-intercept, residual plot and fisher variance ratio (F) were considered in the determination of linearity.

4.2. Recovery

Recovery studies were performed in order to find out how much of the analytes is retrieved during the process of sample preparation. Briefly, quantity of 37.5 mg, 31.25 mg and 25 mg of TRH was spiked separately in the capsule blend corresponding to 80%, 100% and 120% of the concentration of the test solution. These solutions were also spiked with 10 mg, 12.5 mg and 15 mg of KTM. Further, extraction procedure as elaborated in assay section was exercised. The experiment was performed in triplicate. Percent recovery and RSD (%) were calculated.

4.3. Precision

Method repeatability was established by injecting test and standard solution corresponding to 62.5 μg mL^{-1} of TRH and 25 μg mL^{-1} of KTM after independent preparation of each solution. RSD (%) of peak area was calculated. Similarly, % RSD of six independent assay performed on different days was considered. Intermediate precision was carried out by analyzing samples by different analyst on different instrument (Cyberlab).

4.4. Limit of detection and limit of quantitation

The detection and quantitation limits were calculated from calibration curves. The formulae used were LOD = 3.3 σ/S and LOQ = 10 σ/S (where σ is standard deviation of peak area of signal obtained after injection of blank and S is the slope of calibration curve).

4.5. Robustness

Effect of small deliberate changes in detecting wavelength by ±2 nm, column (SMTSAM-C_{18} column, 4.6 × 250 mm, parti-

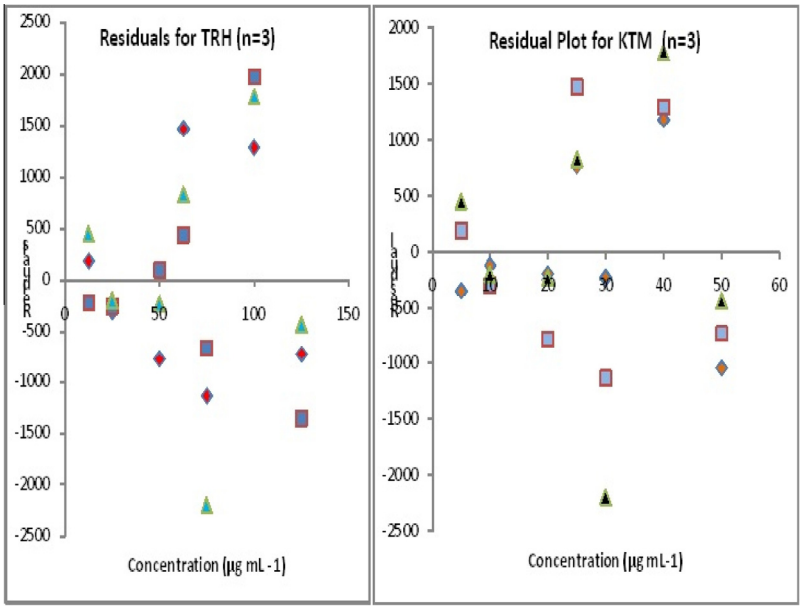


Figure 6 Residual plot for TRH and KTM.

Table 3 Summary of method validation parameters.

S. No.	Parameters		TRH	KTM
1	Linearity (μg mL^{-1})		12.5–125	5–50
	Correlation coefficient		0.998	0.999
	Experimental variance (σ_e^2)		73422.15	938105.2
	Lack-of-fit variance (σ^2_{Lof})		97972.19	2,713,519
	Fisher variance ratio (F) experimental		1.33	2.89
	Fisher variance ratio (F) tabulated		2.96	2.96
2	% Assay (S.D.)		100.2 (1.2)	99.9 (1.3)
3	Recovery (% RSD) $n = 3$	At 80%	100.3 (0.2)	99.8 (0.3)
		At 100%	100.1 (0.1)	99.9 (0.2)
		At 120%	100.3 (0.3)	100.1 (0.3)
4	LOD (μg mL^{-1})		0.48	0.21
5	LOQ (μg mL^{-1})		1.4	0.63
6	Precision	Repeatability ($n = 6$) Mean % assay (RSD)	100.1 (0.12)	99.9 (0.32)
		Intermediate precision ($n = 6$) Mean % assay (RSD)	100.2 (0.43)	100.3 (0.41)
7	Robustness (% assay)	Change in flow rate (0.9 ml/min)	100.2	99.8
		Change in flow rate (1.1 ml/min)	100.3	99.7
		Change detection wavelength 268 nm	99.8	99.9
		Change detection wavelength 272 nm	98.9	99.2
		Change in column	99.5	100.5

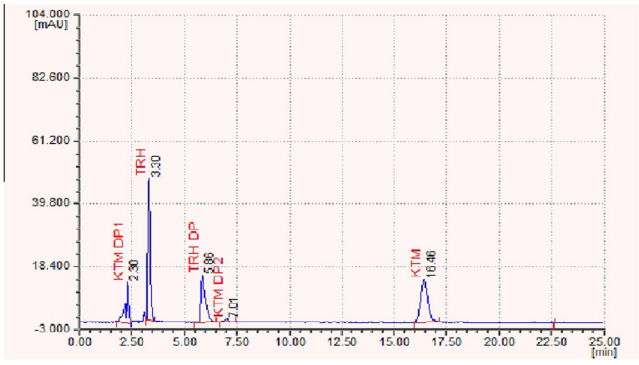


Figure 7 Chromatogram of TRH and KTM showing separation of drugs from their degradation products under acidic stress.

cle size 5 μm) and flow rate by ±0.1 ml/min on assay was checked.

5. Results

5.1. Selection of analytical wavelength

The analytical wavelength selected was 270 nm at which both drugs exhibited substantial absorbance as shown in Fig. 2. The wavelength was selected by acquiring absorption spectra of TRH and KTM.

5.2. Optimization of LC–MS conditions

In order to determine the best conditions for the effective separation, mobile phase containing various ratios of water and methanol was initially used, wherein extensive tailing for TRH was observed. Mobile phase consisting of water: acetonitrile: methanol (53:24:23 v/v/v) with 0.5% formic acid showed good resolution, peak shape and desired elution as shown in Fig. 3.

The ESI source conditions were also optimized to obtain a good signal and high sensitivity. The conditions like drying gas flow, nebulizing gas flow, drying gas temperature and, voltage were optimized to maximize the ionization in the source, response and sensitivity so as to characterize the degradation products. LC–MS chromatograms for TRH and KTM are shown in Fig. 4.

To identify the origin of degradation products, the single drug under stressed condition was introduced into the column. TRH and its degradation products were analyzed at positive ionization mode due to the presence of basic amino group in TRH. KTM and its degradation products were analyzed at negative ionization mode due to the rapid conversion of KTM in to carboxylate ion.

5.3. Stability of sample solution

No significant difference was found in the peak areas of standard solutions of drugs for 8 h (Table 2). This means that the solutions of TRH and KTM are stable for at least 8 h.

5.4. Method validation

The method was found to be selective as the peaks due to both drugs were well resolved from each other and from any other peak due to blank and degradation products. The excipient blend and diluent blank do not show any peak at the retention time of the TRH and KTM as shown in Fig. 5.

The method was found to be linear over the concentration range of 12.5–125 (μg mL^{-1}) for TRH and 5–50 (μg mL^{-1}) for KTM. The correlation coefficients were 0.998 and 0.999 for TRH and KTM respectively. The residuals shown in Fig. 6 do not show any tendency. The calculated experimental value of fisher variance ratio is compared against the critical value of

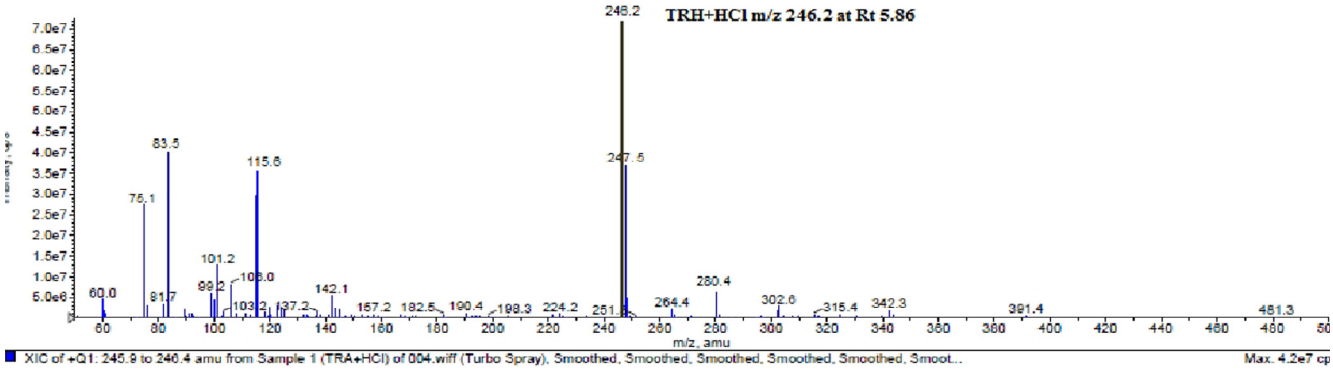


Figure 8 Positive ion ESI LC–MS line spectrum of degradation products of TRH at 5.86 min.

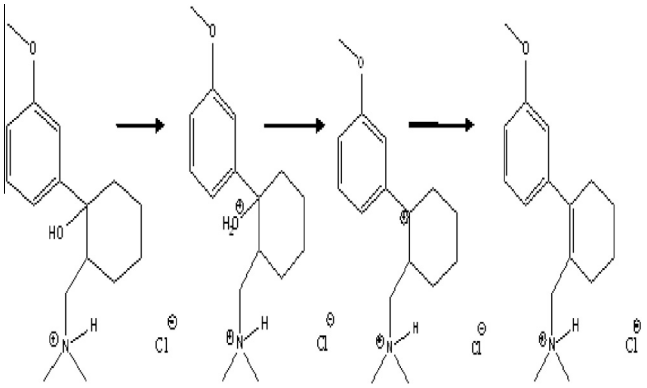


Figure 9 Degradation pathway of TRH under acid hydrolysis (Rt: 5.86 min, m/z: 246.1).

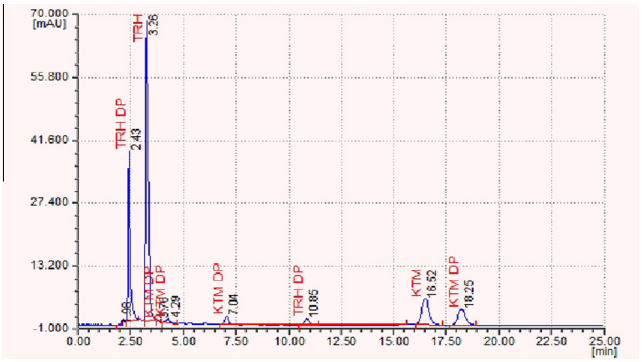


Figure 11 Chromatogram of TRH and KTM after oxidative stress showing separation of drugs from their degradation products.

F found in statistical tables at the 95% confidence level. The tabulated value of *F* is greater the experimental value. This proves the linearity of the method.

The values of % recovery are presented in Table 3. The results indicate, good precision and accuracy was observed. For repeatability study, % RSD of TRH and KTM were found to be 0.12 and 0.32 respectively. The value of % RSD of peak area was less than 2%, indicating good precision of the developed method. The LOD and LOQ of the method is 0.48 and 1.4 μg mL^{-1} respectively for TRH, while they are 0.21 and 0.63 μg mL^{-1} for KTM. These values indicate capability of the method to determine dilute solutions. Robustness study was carried out by performing deliberate changes in detecting wavelength, column and flow rate. This revealed no significant variation in % assay, retention time, tailing factor and theoretical plates. Other method validation parameters are summarized in Table 3.

5.5. Forced degradation studies

5.5.1. Acid stress studies

The comparison of chromatograms obtained with degraded samples and pure samples revealed the presence of one major additional peak in the degraded samples of TRH at retention time of 5.86 min. KTM also showed degradation products at retention time of 2.54 min and 7.01 min. The results are presented in Fig. 7.

TRH and KTM were found to degrade significantly under acidic conditions. The degradation products of TRH and KTM were separately monitored under positive and negative ion modes respectively on LC–MS. TRH showed a degradation product with a mass to charge ratio of 246.2 as shown in Fig. 8. The probable degradation pathway is shown in Fig. 9. The loss of mass 18 is attributed to the elimination of water. KTM also showed two major degradation products at

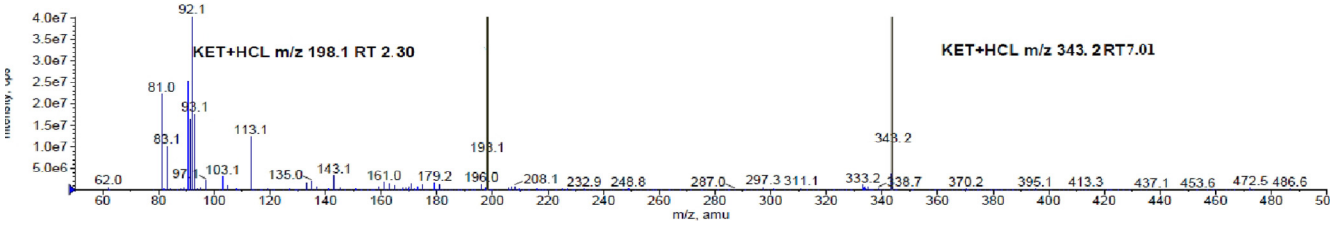


Figure 10 Negative ion ESI LC–MS line spectrum of degradation products of KTM at 7.01 min.

Rt of 2.54 (m/z of 198.1) and 7.01 (m/z of 343.2) as seen in Fig. 10.

5.5.2. *Oxidative stress studies*

The chromatogram of drugs under oxidative stress is shown in Fig. 11. The ESI LC–MS chromatograms of KTM under negative ion mode are presented in Fig. 12. TRH has shown degradation product with m/z value of 280.2 and 195.4. KTM was found to degrade to products with m/z values of 166.1, 306.1, 348.0, 270.1 and 316.0.

Table 4 summarizes the probable structures of the major degradation products of both drugs under acidic and oxidative stress.

6. Discussion

Previous reports have described only physical and chemical compatibility between the two drugs. Lin et al. have assessed compatibility and stability of mixtures of KTM and TRH injection concentrate and diluted infusion solution by HPLC–UV method. They have reported that the mixture is stable for at least seven days at ambient conditions. Stefano et al. have assessed stability TRH injection admixed with selected pain drugs.

The HPLC method carried out in this study was an attempt at developing a simple chromatographic system capable of eluting and resolving TRH and KTM from their degradation products. Preliminary investigations were directed toward carrying out stress studies and developing HPLC method that can resolve all degradation products from drug peak. Parameters assessed were in the nature of mobile phase, its composition and pH. The results show that water: acetonitrile: methanol (53:24:23 v/v/v) with 0.5% formic acid was a suitable mobile phase for the determination of both drugs because of its excellent resolution and appropriate retention time.

TRH and KTM are soluble in water. TRH is soluble in acidic conditions and forms globules under basic conditions. KTM is insoluble at acidic pH. It is soluble under basic and neutral conditions. KTM gives crystalline product after neu-

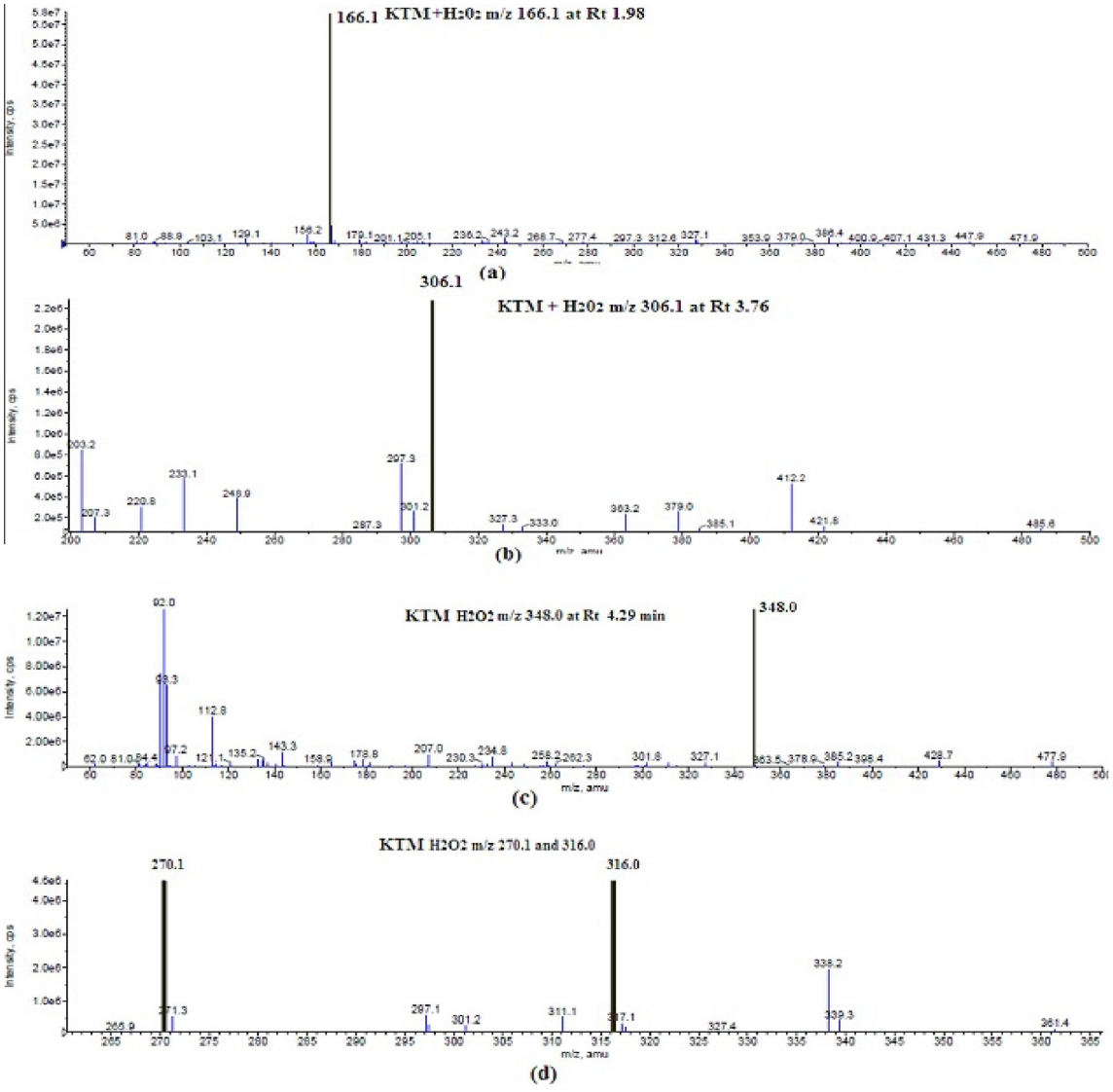
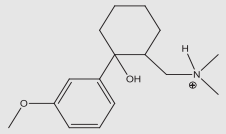
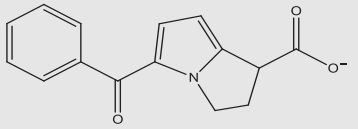
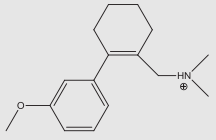
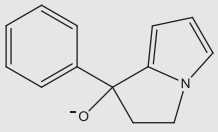
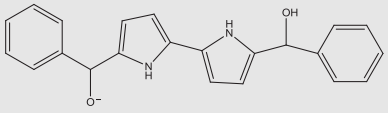
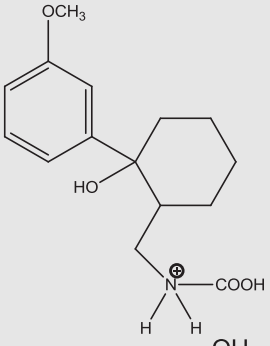
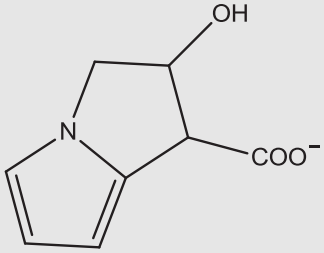
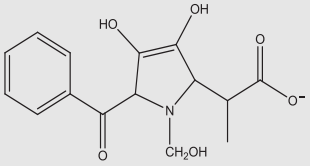


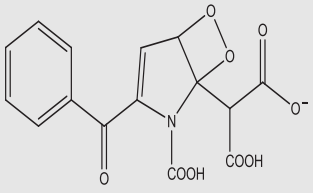
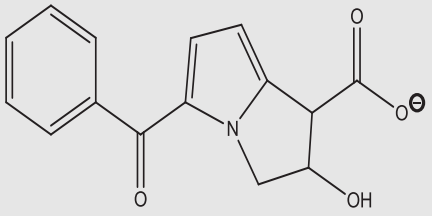
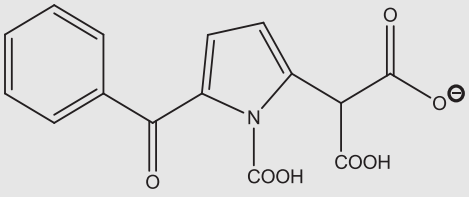
Figure 12 Negative ion ESI LC–MS line spectrum of degradation products of KTM at (a) 1.98 min (b) 3.76 min (c) 4.29 min (d) 7.04 min.

Table 4 Summary of degradation products of TRH and KTM along with the probable structures of some compounds.

Retention time (min)	Origin of peak	Best possible molecular formula	Theoretical mass	Experimental mass	Probable structure
3.02	TRH cation	$C_{16}H_{26}NO_2^+$	265.2	265.2 (M + 1)$^+$	 1-(2-hydroxy-2-(3-methoxyphenyl)cyclohexyl)-*N*,*N*-dimethylmethanaminiu
16.53	KTM Anion	$C_{15}H_{12}NO_3^-$	254.1	254.1 (M − 1)$^+$	 5-benzoyl-2,3-dihydro-1*H*-pyrrolizine-1-carboxylate
5.86	TRH Major DP (Acid hydrolysis)	$C_{16}H_{24}NO^+$	247.2	247.2	 1-(3'-methoxy-3,4,5,6-tetrahydro-[1,1'-biphenyl]-2-yl)-*N*,*N*-dimethylmethanami
4.29	KTM DP (Acid Hydrolysis)	$C_{13}H_{12}NO^-$	198.1	198.1	 1-phenyl-2,3-dihydro-1*H*-pyrrolizin-1-olate
7.01	KTM DP (Acid Hydrolysis)	$C_{22}H_{19}N_2O_2^-$	343.2	343.2	 1*H*,1'*H*-[2,2'-bipyrrole]-5,5'-diylbis(phenylmethanol)
2.43	TRH major DP (oxidation)	$C_{15}H_{22}NO_4^+$	280.2	280.2	
1.98	KTM DP (oxidation)	$C_8H_8NO_3^-$	166.1	166.1	 2-hydroxy-2,3-dihydro-1*H*-pyrrolizine-1-carboxylic acid
3.76	KTM DP (oxidation)	$C_{15}H_{16}NO_6^-$	306.1	306.1	 2-(5-benzoyl-3,4-dihydroxy-1-(hydroxymethyl)-2,5-dihydro-1*H*-pyrrol-2-yl)propanoate

(*continued on next page*)

Table 4 (*continued*)

Retention time (min)	Origin of peak	Best possible molecular formula	Theoretical mass	Experimental mass	Probable structure
4.29	KTM DP (oxidation)	$C_{15}H_{10}NO_9^-$	348.0	348.0	 2-(3-benzoyl-2-carboxy-6,7-dioxa-2-azabicyclo[3.2.0]hept-3-en-1-yl)-2-carboxyacetate
6.10	KTM DP (oxidation)	$C_{15}H_{12}NO_4^-$	270.1	270.1	 5-benzoyl-2-hydroxy-2,3-dihydro-1*H*-pyrrolizine-1-carboxylate
7.04	KTM DP (oxidation)	$C_{15}H_{10}NO_7^-$	316.0	316.0	 2-(5-benzoyl-1-carboxy-1*H*-pyrrol-2-yl)-2-carboxyacetate

tralization. After stress testing, TRH displayed slight precipitation in basic medium which might be due to precipitation of tramadol from its hydrochloride salt under basic conditions. Rest all solutions were clear. All solution dispersed uniformly when diluted with mobile phase to give clear solutions.

This method has demonstrated good precision and recovery. The results were not majorly affected after making deliberate changes in method parameters.

In this study, we have also attempted the characterization of major degradation products of TRH and KTM. During LC–MS study TRH was ionized under positive mode due to the protonation of amino group while KTM was studied under negative ion mode owing to carboxylate ion formation. TRH was found to degrade under acidic conditions with the elimination of water, while KTM has shown higher mass degradation product which may be due to the presence of pyrrole ring which tends to polymerize under acidic conditions. Both drugs were found to degrade significantly under oxidative stress as compared to all stress conditions. KTM has a high oxidation potential as compared to TRH.

Results of degradation studies at different conditions revealed that TRH undergoes extensive degradation at oxidative stress as compared to acid or basic hydrolysis stress. It was found to be stable at neutral stress and to UV light. KTM was also found to degrade more under oxidative stress as compared to its degradation under exposure to UV light followed by acidic stress. After exposure to UV light, it was found to undergo degradation without the appearance of additional peaks. This confirms the generation of nonchromophoric degradation products. It was found to have good stability toward dry heat. No additional peak was displayed in stressed samples of formulation.

This paper thus has focused on stability of both drugs singly and in combination.

7. Conclusion

Quality plays a pivotal role in the determination of safety and efficacy of any pharmaceutical formulation. The presence of potential degradants may alter the properties of formulation. The developed HPLC method is a stability indicating and has been validated for accuracy, precision, robustness and specificity. This method is useful for simultaneous determination of TRH and KTM. Both drugs were found to degrade under oxidative conditions. The structures of major degradation products under acid and oxidative stress have been proposed. The proposed method can also be used for the quantitation of tramadol hydrochloride and ketorolac tromethamine separately in the presence of their degradation products.

Conflict of interest

No conflict of interest.

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